Identification of rod- and cone-specific phosducins in teleost retinas

Yuko Kobayashi, Osamu Hisatomi*, Takunori Satoh, Fumio Tokunaga

Department of Earth and Space Science, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan

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Abstract Phosducin (PD) is a regulatory protein of vertebrate phototransduction cascades. In mammalian retina, it has been thought that only one kind of PD commonly exists in both rods and cones. However, we have found two kinds of PD (OlPD-R and OlPD-C) in the retina of a teleost, medaka (*Oryzias latipes*). In situ hybridization and immunohistochemical analysis demonstrated that OlPD-R and -C are selectively expressed in rods and cones, respectively. The antiserum against medaka PDs recognized two kinds of proteins in bluegill (*Lepomis macrochirus*) retina. These results suggest that rod- and cone-specific PDs exist in teleost retinas, probably creating differences in light adaptation between rods and cones.

Key words: Phosducin; Photoreceptor cell; Retina; Teleost; Medaka (*Oryzias latipes*)

1. Introduction

In vertebrate phototransduction cascades, there are mechanisms to regulate the efficiency of signal transduction. Several proteins, phosducin (PD) being one, play roles in these regulations [1]. PD is a soluble phosphoprotein abundant in vertebrate retinas and developmentally related pineal glands [2–4]. In rods, photo-activated rhodopsin stimulates the photoreceptor-specific heterotrimeric G-protein, transducin (Gtαβγ), to exchange GDP for GTP. Then the GTP-conjugated α subunit of Gt (Gtα-GTP) dissociates from the βγ subunit (Gtβγ) and activates cGMP-phosphodiesterase to lead to the hyperpolarization of rods. GTP bound to Gtα is hydrolyzed by the intrinsic GTPase activity of Gtα, and the consequent inactive Gtα-GDP reassociates with Gtβγ to form the initial Gtαβγ. It has been suggested that unphosphorylated PD forms a complex with Gtβγ in the light-adapted state, and then prevents the reassociation of Gtα with Gtβγ by blocking their interaction face and by translocating Gtβγ away from the photoreceptor disk membrane [5–7]. In essence, by interacting with Gtβγ, PD regulates the amount of active Gtα-GTP produced by a photo-activated rhodopsin.

The affinity of PD to Gtβγ is determined by its phosphorylation state. When a serine residue (S73 of bovine PD) is phosphorylated by cAMP-dependent protein kinase (PKA), PD dissociates from Gtβγ [8–10]. In rod outer segments, the activity of PKA is thought to be regulated by the intracellular concentration of cAMP produced by a Ca^{2+}-dependent adenylate cyclase [11]. Intracellular Ca^{2+} concentration is high in the dark-adapted state and low in the light-adapted state [12,13]. That is, light adaptation accelerates the dephosphorylation of PD, which inhibits the formation of Gtαβγ and reduces the photo-sensitivity of photoreceptor cells [3,14].

Although many researchers have studied mammalian PDs, little is known about non-mammalian PDs. The purpose of our study is to find and investigate PDs in lower vertebrate retinas. In this paper, we report two kinds of cDNAs encoding putative PDs (OlPD-R and OlPD-C) of a teleost (medaka, *Oryzias latipes*). Unlike previous reports, which have stated that only one kind of PD exists in mammalian photoreceptor cells [2,15], our results indicate that OlPD-R and -C are selectively expressed in rods and cones, respectively. Further, the antisera against medaka PDs recognized plural proteins in bluegill retina, suggesting that many teleosts have rod- and cone-specific PDs in their retinas.

2. Materials and methods

2.1. Fish

Medaka (*O. latipes*) of 2–3 cm in length were obtained from a local supplier, and bluegill (*Lepomis macrochirus*) of about 15 cm in length were obtained from a local pet shop. Fish were kept in indoor aquaria on a daily cycle.

2.2. Isolation of cDNAs encoding putative medaka PDs

Two kinds of oligonucleotide mixtures were prepared: PD-F1 (5′-GCGAATTCAA(A/G)GGNGTNAT(A/T/C)AA(C/T)TGG-3′; KGVINDW) for sense priming, and PD-R1 (5′-GCGGATCCCA(G/A)AA(T/C)TTNACNATNGG(G/A)TA-3′; YP(M/I)VKFC) for antisense priming. These amino acid sequences correspond to the conserved mammalian PD sequences reported so far. cDNA fragments encoding putative medaka PDs were amplified by polymerase chain reaction (PCR) and a medaka retinal cDNA library (containing 10^6 independent clones) was screened using the amplified fragments as probes. Library screening and sequencing procedures were the same as described previously [16]. The nucleotide sequence data have been deposited to the DDBJ/GenBank/EMBL nucleotide sequence databases with accession numbers AB014466 (OlPD-R) and AB014467 (OlPD-C). The amino acid sequence data used in the present analyses were taken from the PIR database, with the following accession numbers: bovine phosducin (A38379) and rat phosducin-like protein PhLP (long splice form) (A58928).

2.3. In situ hybridization

cDNA fragments were cloned into a pGEM-3Zf(+) plasmid vector (Promega) and linearized with appropriate endonucleases. Antisense cRNA riboprobes (about 500 bases in length) were synthesized by run-off transcription from the SP6 or T7 promoter with digoxigenin-UTP, as recommended in the manufacturer's protocol (Boehringer Mannheim). Preparation of medaka retinal cryosections and methods for in situ hybridization were as described previously [16,17]. The hybridization signals were visualized using a nucleic acid detection kit (Boehringer Mannheim), and detected with Nomarski optics (OLYMPUS-BX50).

*Corresponding author. Fax: (81)-6-6850 5480.
E-mail address: hisatomi@ess.sci.osaka-u.ac.jp (O. Hisatomi).

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2.4. Preparation of the antisera against medaka PDs

cDNA fragments encoding the C-terminal region of OlPD-R (E150–E235) and OlPD-C (E153–E236) were amplified and cloned into a pQE-16 expression vector (Qiagen) designed to express as DHFR fusion proteins with a histidine hexamer tail. *Escherichia coli* cells (SG13009, Qiagen) were transformed by the plasmids, and the expression of recombinant proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration 1 mM). The proteins were isolated using a Ni-NTA column as recommended in the manufacturer's protocol (QIA purification system, Qiagen), and then used as antigens for mice immunization to produce antisera (anti-RC and anti-CC). Before use, each anti-RC and anti-CC antisera were preabsorbed with the other antigen at 4°C overnight to remove cross-reactivity. Full-length OlPD-R with a histidine hexamer tail was also expressed with the aid of a pQE-60 expression vector (Qiagen), and isolated with the same procedure as described above. The antiserum (anti-R) raised against this recombinant protein was used without preabsorption.

2.5. Western blotting and immunohistochemistry

Fish retinas were homogenized in the sodium dodecyl sulfate (SDS) sample buffer and briefly sonicated. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to PVDF membrane (Bio-Rad), as described by Imanishi et al. (1999) in the presence of 15% methanol for 1 h [18]. Membranes were blocked for 2 h with 3% BSA and 0.1% Tween-20 in phosphate-buffered saline (PBS), and then incubated for 1 h with a 1000-fold dilution of the antisera in TBS buffer (150 mM NaCl and 2 mM KCl, 50 mM Tris–HCl, pH 7.4) containing 0.1% Tween-20. For control samples, normal mouse serum was used as the primary antibody. Membranes were incubated with biotin-conjugated anti-mouse IgG antibodies (KPL, 0.2 μg/μl diluted in TBS), and reacted with peroxidase-conjugated avidin–biotin complex, as recommended in the manufacturer's protocol (ABC Kit, Vector). Antibody binding was visualized with diaminobenzidine (DAB). All procedures were carried out at room temperature.

Retinal cryosections of medaka and bluegill were prepared as described in Section 2.3. Cryosections were treated with proteinase K, blocked for 1 h at room temperature with 3% BSA in PBS containing 0.1% Tween-20, and reacted for 30 h at 4°C with the primary antisera (1:100 diluted in PBS), followed by incubation for 20 h at 4°C with peroxidase-conjugated anti-mouse IgG secondary antibodies (ICN, 1:100 diluted in PBS). Signals were visualized with DAB developing solution and observed with Nomarski optics (OLYMPUS-BX50).

3. Results

3.1. Isolation of cDNAs encoding medaka PDs

Two kinds of cDNA fragments encoding the putative medaka PDs, OlPD-R and OlPD-C, were amplified by PCR from a medaka retinal cDNA pool, and clones including the complete coding regions were isolated from a medaka retinal cDNA library [16]. OlPD-R and -C cDNAs appeared to encode proteins of 235 and 236 amino acids, respectively (Fig. 1). The deduced amino acid sequences of medaka PDs show 72% identity with each other, approximately 60% identity to those of mammalian PDs, but less than 50% identity to that of a phosducin-like protein (PhLP) of rat [19]. It is therefore likely that both OlPD-R and -C are medaka PDs, but not PhLP. The molecular masses and p*I* values predicted from the amino acid sequences also showed agreements with those of mammalian PDs [2,3]. Residues, which have been suggested as playing important roles in the function of mammalian PDs, were found in each sequence, a highly conserved 11 contiguous amino acid loop region (TGPKGVINDWR) that is crucial for interaction with Gtβγ [6], and a consensus sequence for phosphorylation by PKA (RKMS) [20] (Fig. 1). These results imply that OlPD-R and -C play roles, similar to those of mammalian PDs, in medaka photoreceptors, that is, OlPD-R and -C are functional medaka PDs.

3.2. In situ localization of medaka PD mRNAs

Medaka photoreceptor cells can be categorized morphologically into five types: rods, principal and accessory members of double cones, long single cones and short single cones [16]. Distributions of the medaka PD mRNAs in the retina were investigated by in situ hybridization. Digoxigenin-conjugated OlPD-R cRNA probe only recognized the outer nuclear layer in radial sections of medaka retina (Fig. 2a). Signals were localized in the cell bodies and the myoid regions of rods, but were not found in cones (Fig. 2b). A similar staining pattern was observed with in situ hybridization using medaka

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RAT-PhLP                                1 MTTLDDKLLGEKLQYYYSTSEDEDSDHEDKDRGRGAP  37

                                        PD-F1
OlPD-R     1  MSSVVQEEEELP---ANHTGPKGVINDWRRFK-LDSVD-QNVPQRKRELLRQMS----------  49
OlPD-C     1  MSNKLIELEEN----ATHTGPKGVINDWRRFK-LESMDRENLPSAKKELLRQMS---------S  50
BOVINE     1 MEKAKSQSLEEDFEGQASHTGPKGVINDWRKFK-LESEDSDSVAHSKKEILRQMS---------S  55
RAT-PhLP  38 ASSSTPAEAELAGEGISVNTGPKGVINDWRRFKQLETEQREEQCREMERLIKKLSMSCRSHLDEE 102
                           **********

OlPD-R    50 --NPRD-DDKERVNRKMSVQEYEMIQ--DEDERCLKKYRKQCMQEMHERLSFGPKFECVHELESG 109
OlPD-C    51 PNKSKD-DSRANLNRKMSVQEYELLK--EEDEGCLKKYRRQCMEEMHNKLSFGPRFEGVHDLDSG 112
BOVINE    56 PQSRDDKDSKERFSRKMSVQEYELIHKDKEDENCLRKYRRQCMQDMHQKLSFGPRYGFVYELESG 120
RAT-PhLP 103 EEQQKQKDLQEKISGKMTLKECGMMDKNLDDEEFLQQYRKQRMDEMRQQLHKGPQFKQVLEIPSG 167
                                                    PD-R1
OlPD-R   110 EAFLEVIEKEHRLTLVVVNIYQQDVKGCEQMNSCLDCLATEYPTVKFCRIDAVATGAAERFSSEV 174
OlPD-C   113 EAFLEVIEKEHHSTVVVVHIYKIGIKGCEQLNSCLDCLATEYPTVKFCRIDAVSSGAAERFSDEY 177
BOVINE   121 EQFLETIEKEQKITTIVVHIYEDGIKGCDALNSSLICLAAEYPMVKFCKIKASNTGAGDRFSSDV 185
RAT-PhLP 168 EGFLDMIDKEQKSTLIMVHIYEDGVPGTEAMNGCMICLAAEYPTVKFCRVRSSVIGASSRFTRNA 232


OlPD-R   175 LPTLLVYKSGELLGNFLSITKHFNEEFFATDVEAFLNEYGLLPEKEFAACADEEDDAANVE     235
OlPD-C   178 LPTLLVYKAGELLGNFLSITQHLSEEFFATDVEAFLNSYGLLPEKELPKLEDEE--ENDVE     236
BOVINE   186 LPTLLVYKGGELLSNFISVTEQLAEEFFTGDVESFLNEYGLLPEKEMHVLEQTNMEEEDME     246
RAT-PhLP 225 LPALLIYKAGELIGNFVRVTDQLGEDFFAVDLEAFLQEFGLLPEKEVLVLTSVRNSATCHSEDSD 297

RAT-PhLP 298 LEID 301
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Fig. 1. The deduced amino acid sequences of OlPD-R and OlPD-C arranged with those of bovine PD and rat PD-like protein, PhLP. Arrows indicate the positions corresponding to the primers used for amplification of medaka PD cDNA fragments. Asterisks represent the highly conserved loop region, and shaded boxes shows the residues that were suggested to be crucial for interaction with Gtβγ (see text) [6]. The box indicates the recognition sequence of PKA and the arrowhead indicates the putative site for phosphorylation. A broken line shows the C-terminal regions that were used for the expression of recombinant antigens to produce anti-RC and anti-CC antisera.

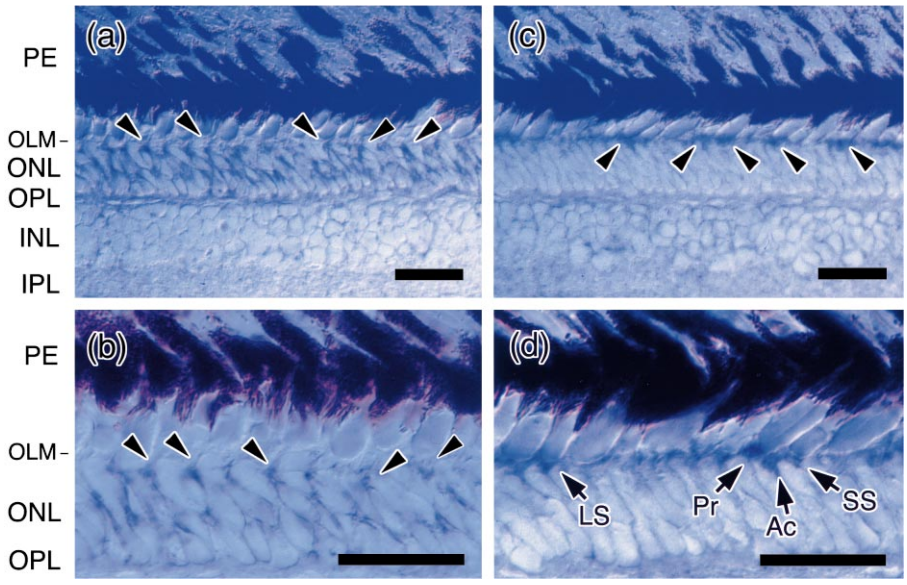

Fig. 2. Localization of OlPD-R (a and b) and OlPD-C (c and d) mRNAs in radial sections of medaka retina. b and d are higher magnification images. Arrowheads indicate the hybridization signals. Abbreviations: PE, pigment epithelium; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; principal (Pr) and accessory (Ac) members of double cone; LS, long single cone; SS, short single cone. Scale bar = 20 μm.

rhodopsin cRNA as a probe [16], so we concluded that OlPD-R was expressed exclusively in rods. Fig. 2c and d show the localization of OlPD-C mRNA. Hybridization signals were found only around the outer limiting membrane, and localized in the myoid regions and inner segments of cones. The localization of the signals was quite similar to those of opsins, an arrestin, a kinase and a guanylate cyclase expressed in medaka cones [16,21–23], suggesting that OlPD-C is selectively expressed in cones, but not in rods. Cones negative to the OlPD-C cRNA probe were not identified in our experiments.

3.3. Western blot analysis

Three kinds of antisera were prepared against full-length OlPD-R (anti-R), C-terminal region of OlPD-R (anti-RC) and OlPD-C (anti-CC). To avoid cross-reactivity, anti-RC and anti-CC were preabsorbed with the C-terminal regions

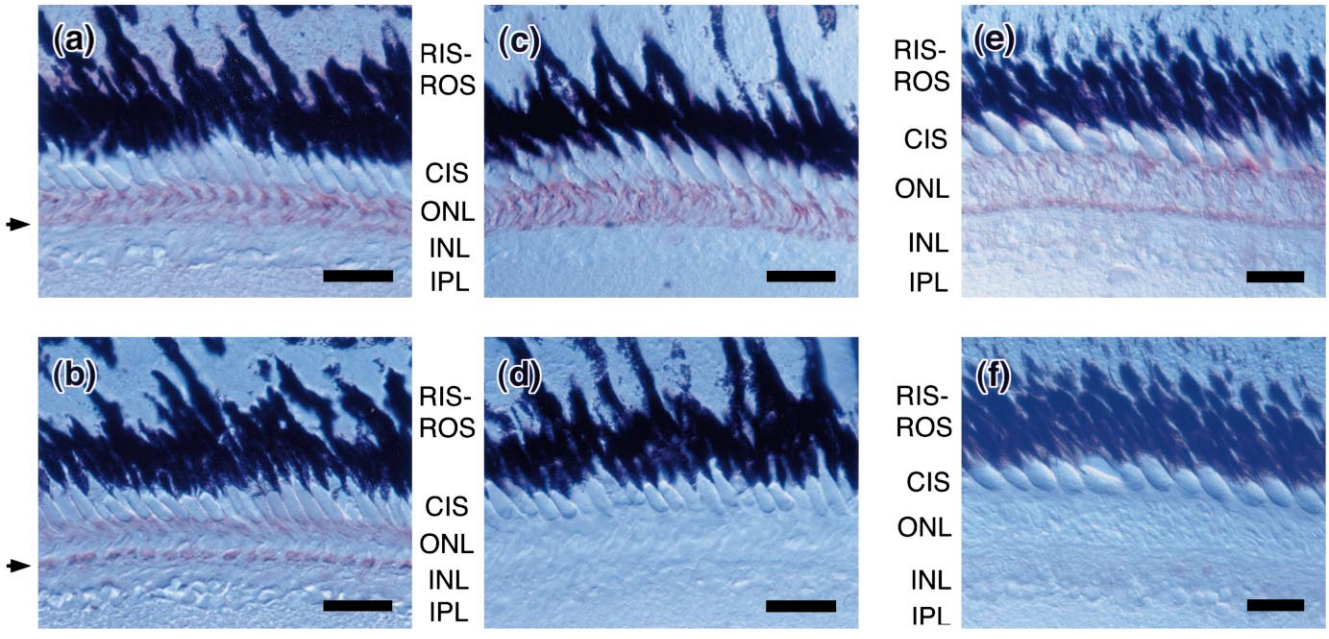

Fig. 3. Reactivity of (a) anti-RC, (b) anti-CC and (c) anti-R antisera in radial sections of medaka retina. Arrows indicate signals found in the synaptic termini of photoreceptor cells. d: Control experiment using normal mouse serum instead of anti-PD antisera. Reactivity of (e) anti-R and (f) normal serum (control) in a radial section of bluegill retina. Abbreviations: RIS–ROS, rod inner–outer segment; CIS, cone inner segment; and others as in Fig. 2. Scale bar = 20 μm.

of OlPD-C and OlPD-R, respectively. Anti-R was used without preabsorption.

Western blot analysis of the medaka retinal homogenate demonstrated that anti-R recognized two bands, one of 34 kDa and one of 32 kDa (Fig. 4a, lane 1). The 34 kDa and 32 kDa proteins were selectively reacted with anti-RC (lane 2) and anti-CC (lane 3), respectively. These apparent molecular masses are very close to those of mammalian PDs (33 kDa) estimated from the mobility on SDS–PAGE [2,3]. We therefore concluded that the 34 kDa and 32 kDa proteins were OlPD-R and -C, respectively, and that anti-R reacted with both OlPD-R and -C.

Two kinds of PKA substrate proteins, pp35 and pp33 (with apparent molecular masses on SDS–PAGE of 35 kDa and 33 kDa, respectively), were detected in the rod inner–outer segment (RIS–ROS) homogenate of a teleost, green sunfish (*Lepomis cyanellus*) [24]. Since both of them were recognized by an anti-bovine PD antibody, it was speculated that these proteins were PD homologs. However, pp33 shows certain differences in its phosphorylation pattern and in its sensitivity for proteases from those of mammalian PDs [25]. We tested the retinal homogenate of a teleost, bluegill (*L. macrochirus*), that belongs to the same genius as the green sunfish. Anti-R recognized bands at 35 kDa and 33 kDa with a faint band at about 30 kDa in bluegill retina (Fig. 4b, lane 1). However, the 30 kDa band was detected even in the control sample using a normal serum instead of anti-R (Fig. 4b, lane 2). Preabsorbed anti-RC and anti-CC did not show reactivity with either the 35 kDa or 33 kDa bands (data not shown), may be due to the loss of components which could react with the conserved amino acid sequences in the C-terminal region of OlPD-R and -C.

3.4. Immunohistochemistry

The localization of PDs in light-adapted medaka and bluegill retinas was investigated by immunohistochemical analysis using anti-RC and anti-CC antisera, and non-preabsorbed anti-R antiserum. Anti-RC mostly recognized the outer nuclear layer (ONL). It appears that heavy signals were localized in the myoids and cell bodies of rods, while faint signals were observed in the ROS–RIS (Fig. 3a). In contrast, anti-CC recognized the inner and outer sides of ONL. Signals were found in the myoids, cell bodies and synaptic termini of cones (Fig. 3b). The staining pattern for anti-R corresponds to those for anti-RC plus anti-CC (Fig. 3c), probably showing the localization of both OlPD-R and -C. No significant difference in the reactivity of each antiserum was observed between light- and dark-adapted medaka photoreceptors (data not shown). The anti-R immunoreactivity in bluegill retina was similar to that in medaka retina (Fig. 3e), with no signals being detected in the rest of the retina. It is likely that both immunopositive 35 kDa and 33 kDa proteins are PD homologs of bluegill, and are expressed in photoreceptor cells. In control experiments using a normal mouse serum instead of anti-PD antisera, no specific labeling was observed in medaka (Fig. 3d) and bluegill (Fig. 3f) retinas.

The localization of medaka PDs in the inner segments and cell bodies of light-adapted photoreceptor cells corresponds to findings in previous reports that mammalian PDs mostly exist around the inner segments of photoreceptor cells [2,26,27]. Further, it has been reported that an anti-Gtβ monoclonal antibody showed a similar staining pattern in light-adapted mouse retina [26,27]. Because PDs form complexes with Gtβγ, it is likely that PDs are co-localized with Gtβγ in light-adapted retina.

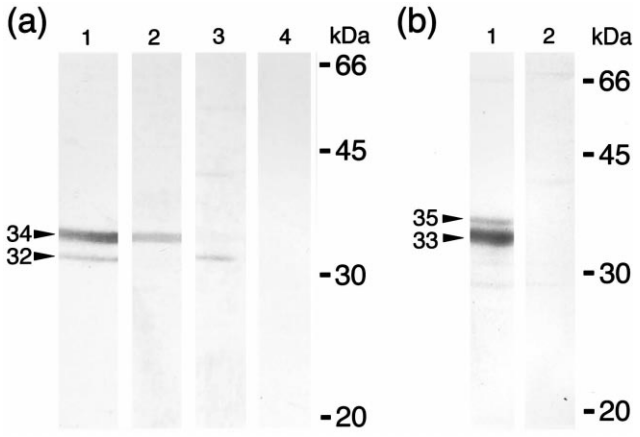

Fig. 4. a: Western blot analysis of medaka retinal homogenate. Anti-R antiserum recognized proteins with apparent molecular masses of 34 kDa and 32 kDa (lane 1). Preabsorbed anti-RC (lane 2) and anti-CC (lane 3) recognized the bands of 34 kDa and 32 kDa, respectively. For control experiments, a normal mouse serum was used instead of the primary antisera (lane 4). b: Western blot analysis of bluegill retinal homogenates. Anti-R recognized bands with molecular masses of 33 kDa and 35 kDa (lane 1). Control experiment using a normal mouse serum instead of the anti-R (lane 2).

4. Discussion

Our observations are clearly indicative of the existence of rod- and cone-specific PDs in medaka retina. In addition to medaka PDs, anti-R antisera recognized two kinds of proteins, with apparent molecular masses of 35 kDa and 33 kDa, in bluegill retinal homogenate, possibly corresponding to pp35 and pp33 in green sunfish retina. Recently, it has been reported that two kinds of partial cDNA fragments (for example BF937751 and BF938012 in DDBJ/GenBank/EMBL) which are closely related to OlPD-R and -C, respectively, are present in a zebrafish cDNA library. These results suggest that many teleosts have plural PDs in their retinas.

It has previously been thought that only one kind of PD commonly exists in mammalian rods and cones, because only one kind of cDNA was cloned from retina, and anti-PD antibodies equally recognized these photoreceptor types [2,15,28]. The one exception was that an anti-bovine PD antibody showing different reactivity between blue and green cones, has been demonstrated in ground squirrel retina [29]. However, it remains unclear whether or not mammals have just one PD, and why it is that teleosts have two kinds of PDs. Rods and cones are known to have similar isozymes of phototransduction proteins, and it is believed that the molecular properties of these isozymes influence the physiological responses of rods and cones [22,30,31]. Gaudet et al. suggested the PD residues that directly contact with Gtβγ from the crystal structure analysis of mammalian PD–Gtβγ complex [6]. Most of these residues have been conserved between medaka and bovine PDs. In these residues, only two conservative substitutions (N14 and N60 of OlPD-R) are found between OlPD-R and bovine PD sequences, whereas two conservative (T13 and N63 of OlPD-C) and two non-conservative substitutions (N61 and S215 of OlPD-C) are found between OlPD-

C and bovine PD sequences (see Fig. 1). It is possible that these non-conservative substitutions of OlPD-C affect the interaction with Gβγ and generate some difference in the sensitivity and adaptation between medaka rods and cones [32,33].

Recently, several studies have indicated that mammalian PD can interact with various proteins other than Gβγ, suggesting that PD plays some additional roles in photoreceptor cells. Zhu and Craft have reported that PD interacts with the cone–rod homeobox protein (CRX), and probably participates in transcriptional regulation [34,35]. Nakano et al. have pointed out that PD binds to the 14-3-3 protein in rat photoreceptor inner segments in a light-dependent manner, and proposed a hypothesis that PD is involved in the regulation of photoreceptor sensitivity [36]. Because the reactivity of anti-medaka PD antisera was observed out of the outer segments, it is possible that teleost PDs also play such undefined roles in addition to the regulation of phototransduction. If it sees from this viewpoint, it is interesting that Pagh-Roehl et al. mentioned that pp33 and pp35 of green sunfish might be connected with the mechanisms of retinomotor movement in photoreceptor cells [25]. We found that anti-RC and anti-CC markedly reacted with the synaptic termini of photoreceptor cells (see Fig. 3a,b). It has been reported that mammalian PDs and several phototransduction proteins, such as Gtβ, arrestin and RGS9, were localized at the synaptic termini of photoreceptor cells [26,27,37,38]. Moreover, several kinds of G-protein-coupled receptors exist in this region [39,40]. Because mammalian PDs are known to be able to regulate various G-protein subtypes (Gs, Gi, Go, etc.) in addition to Gt [41–43], these teleost PDs may interact with and regulate an unknown G-protein-coupled signaling pathway at the synaptic terminal region.

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